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Attachment Characteristics and Involvement of Integrins in Adhesion of Breast Cancer Cell Lines to Extracellular Bone Matrix Components

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SUMMARY: Evidence is mounting that changes in the ability of cancer cells to adhere to extracellular matrices play a decisive role in metastatic spread. The mechanism underlying the preference of breast cancer cells to metastasize to bone is, however, poorly understood. We investigated the expression and involvement of integrin adhesion receptors in the adhesion of breast cancer cells to bone matrix (constituents) in two in vitro attachment assays using RGD peptides and anti-integrin antibodies. Breast cancer cells adhered rapidly to extracellular bone matrix. Adhesion of most cells to vitronectin, fibronectin, thrombospondin, osteopontin, and the fairly bone-specific bone sialoprotein was inhibited by the 200 μg/ml GRGDS peptide. These data suggest that integrin adhesion receptors can modulate the attachment of breast cancer cells to bone matrix molecules. In accordance with these findings, we found that α_1-$\alpha_5(\beta_1)$ and $\alpha_v(\beta_3)$ integrins were expressed by mammary carcinoma cells. Highly tumorigenic MDA-MB-231 cells, which form osteolytic metastases in vivo, expressed relatively high levels of $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_3$ integrins, when compared to MCF-7, T47D, and ZR75-1 breast cancer cells. Addition of function-blocking anti-$\alpha_2\beta_1$, -$\alpha_3\beta_1$, -$\alpha_5\beta_1$, and -$\alpha_v\beta_3$ antibodies significantly inhibited the adhesion of MDA-MB-231 breast cancer cells to bone matrices. In conclusion, our data suggest a possible role for β_1 and β_3 integrin subfamily members in the establishment of skeletal metastases in advanced breast cancer patients. Clearly, functional evidence is required to understand the mechanisms involved in the development of skeletal metastases in breast cancer patients. (*Lab Invest 1997, 77:665–675*).

Tumor growth and formation of metastases depend largely on alterations in interactions between malignant cells and their microenvironment (Albelda, 1993; Juliano, 1993; Maemura and Dickson, 1994; Varner and Cheresh, 1996; Zetter, 1993). These involve changes in the ability of cancer cells to adhere to adjacent cells and surrounding extracellular matrices, which are mediated by specific cell-surface molecules (adhesion receptors) and are believed to play a decisive role in metastatic spread (Albelda, 1993; Juliano, 1993; Maemura and Dickson, 1994; Varner and Cheresh, 1996; Weaver et al, 1997; Zetter, 1993). Differential expression and/or activation of adhesion receptors on tumor cells, especially those of the integrin family, have been implicated in tumor progression and metastatic spread (Albelda, 1993; Juliano, 1993; Maemura and Dickson, 1994; Varner and Cheresh, 1996; Weaver et al, 1997; Zetter, 1993).

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The skeleton is the most common site of distant metastases from breast cancer. Bone metastasis is thought to be a selective process (Coleman and Rubens, 1987; Elte et al, 1986; Orr et al, 1993; Paterson, 1987; Rosol and Capen, 1992; Yoneda et al, 1994), but little is known about the mechanisms that account for the apparent affinity of breast cancer for bone tissue. In a limited number of studies, it has been suggested that expression of integrin subunits may be altered at skeletal sites (Kitazawa et al, 1995; Liapis et al, 1996; Matsuura et al, 1996). It may, therefore, be that breast cancer cells have or acquire high-affinity receptors for extracellular bone matrix (molecules).

Bone matrix contains a unique mixture of extracellular connective tissue proteins, which are predominantly produced by cells of the osteoblast lineage (Bianco et al, 1991; Fisher, 1992; Gehron Robey, 1989; Gehron Robey and Termine, 1985; Gehron Robey et al, 1989; Grzesik and Gehron Robey, 1994; Ingram et al, 1993; Mintz et al, 1993; Oldberg et al, 1986, 1988; Preissner, 1991; Reinholt et al, 1990; Termine, 1993; Weiss and Reddi, 1981). Collagenous proteins (type I collagen fibers) represent 85% to 90% of total bone protein, whereas noncollagenous proteins comprise the remaining 10% to 15%. On a mole-to-mole basis, however, bone cells synthesize and secrete as many molecules of noncollagenous proteins as collagen

(COL) (Termine, 1993). Noncollagenous proteins are subdivided into four groups: (a) cell attachment proteins, (b) proteoglycans, (c) γ-carboxylated (gla) proteins, and (d) growth-related proteins (Gehron Robey, 1989; Gehron Robey and Termine, 1985; Gehron Robey et al, 1989; Grzesik and Gehron Robey, 1994). The list of proteins with cell attachment activity that can be isolated from mineralized bone matrix is best headed by the those containing RGD (Arg-Gly-Asp), a cell attachment recognition-consensus sequence for several adhesion receptors of the integrin family (Pierschbacher and Ruoslahti, 1984). There are at least six of these RGD-containing proteins in bone: type I collagen (COL-I), thrombospondin (TSP), fibronectin (FN), vitronectin (VN), osteopontin (OPN), and bone sialoprotein (BSP) (Bianco et al, 1991; Fisher, 1992; Gehron Robey, 1989; Gehron Robey and Termine, 1985; Gehron Robey et al, 1989; Grzesik and Gehron Robey, 1994; Oldberg et al, 1986, 1988; Reinholt et al, 1990; Termine, 1993). Any of these alone or in combination may, therefore, serve as a unique "glue" for highly specialized normal cells (eg, osteoclasts and osteoblasts) as well as osteotropic tumor cells (eg, breast cancer cells). We have recently shown that breast cancer cells adhere strongly to immobilized synthetic peptides, which encompass the RGD recognition motif and flanking regions of the fairly bone-specific BSP (van der Pluijm et al, 1996a). These results suggest that integrins are involved in adhesion of breast cancer cells to extracellular bone matrices.

In this study, we have examined the attachment of breast cancer cells to extracellular bone matrices (trabecular bone, matrix deposited by bone cells) and adhesion proteins that are naturally found in bone. The expression of integrins and their involvement in adhesion of breast cancer cells to various substrates were studied using RGD peptides and function-blocking anti-integrin antibodies.

Results

Four breast cancer cell lines (MCF-7, T47D, ZR75-1, and MDA-MB-231) were tested for their ability to adhere to various immobilized extracellular matrices (Table 1). MCF-7 cells adhered strongly to BSP, followed by VN, serum-free conditioned medium (SFCM) of human bone cells, COL-I, FN, OPN, TSP, and laminin (LAM) (Table 1), in this particular order. T47D tumor cells adhered to the tested substrates in a similar but not identical fashion (Table 1). Again, these tumor cells attached rapidly and effectively to BSP, followed by VN, TSP, SFCM, LAM, COL-I, FN, and OPN. Consistent with MCF-7 and T47D, ZR75-1 cells were found to settle and spread easily on BSP (Table 1), followed by VN, SFCM, LAM, COL-I, FN, OPN, and TSP. MDA-MB-231 breast cancer cells strongly adhered to SFCM of human bone cells, followed by COL-I, LAM, FN, VN, BSP, TSP, and OPN (Table 1).

The involvement of integrins in the adherence of the four cell lines to substrates was tested in the presence of the synthetic RGD-containing peptide GRGDS (Table 1). Adhesion of all tested cell lines to OPN and BSP was completely inhibited by GRGDS, whereas adhesion to COL was not blocked by GRGDS, although the relative inhibition of cellular adhesion to VN, FN, TSP, LAM, and SFCM by GRGDS varied among the cell lines tested. Representative examples of the attachment of MCF-7, T47D, and ZR75-1 cells to SFCM and BSP are shown in Figure 1.

The data obtained in the attachment experiments suggested involvement of integrins that recognize RGD motifs in adhesion of the cancer cells to several components of the extracellular matrix. However, the lack of inhibitory effects of RGD peptides on adhesion of breast cancer cells to SFCM of human bone cells does not exclude the contribution of integrins that recognize adhesion motifs other than RGD. To determine the putative role of these other integrins, we have

Table 1. Attachment Characteristics of Various Breast Cancer Cell Lines[a] to Extracellular Bone Matrix[b] and Its Individual Molecules[c,d]

	MCF-7		T47D		ZR75-1		MDA-MB-231	
GRGDS	−	+	−	+	−	+	−	+
BSA	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
SFCM	70 ± 5	65 ± 7	140 ± 3	179 ± 11	182 ± 12	130 ± 11	160 ± 15	188 ± 19
OPN	27 ± 3	0 ± 0[e]	62 ± 17	0 ± 0[e]	65 ± 5	0 ± 0[e]	30 ± 0	0 ± 0[e]
BSP	170 ± 25	0 ± 0[e]	273 ± 25	0 ± 0[e]	385 ± 20	0 ± 0[e]	75 ± 5	0 ± 0[e]
FN	30 ± 2	4 ± 1[e]	80 ± 5	20 ± 5[e]	90 ± 8	91 ± 16	100 ± 5	45 ± 5[e]
VN	100 ± 3	6 ± 1[e]	190 ± 20	25 ± 3[e]	200 ± 30	20 ± 5[e]	88 ± 3	28 ± 2[e]
COL-I	45 ± 4	53 ± 2	125 ± 7	100 ± 10	165 ± 5	150 ± 5	100 ± 4	105 ± 15
TSP	27 ± 2	10 ± 2[e]	170 ± 4	60 ± 2[e]	60 ± 17	25 ± 2[e]	60 ± 1	62 ± 5
LAM	20 ± 5	55 ± 7	125 ± 5	140 ± 2	190 ± 14	90 ± 7[e]	101 ± 12	84 ± 8

[a] MCF-7, T47D, ZR75-1, and MDA-MB-231.
[b] SFCM, Serum-free conditioned medium of normal human trabecular cells.
[c] OPN, Osteopontin; BSP, Bone sialoprotein; COL-I, Type I collagen; FN, Fibronectin; VN, Vitronectin; TSP, Thrombospondin; LAM, Laminin.
[d] Cell lines were examined in the presence or absence of 200 μg/ml GRGDS peptide. 200 μg/ml GRADS peptide was used as a negative control.
[e] $p = \leq 0.05$.
± = SEM.

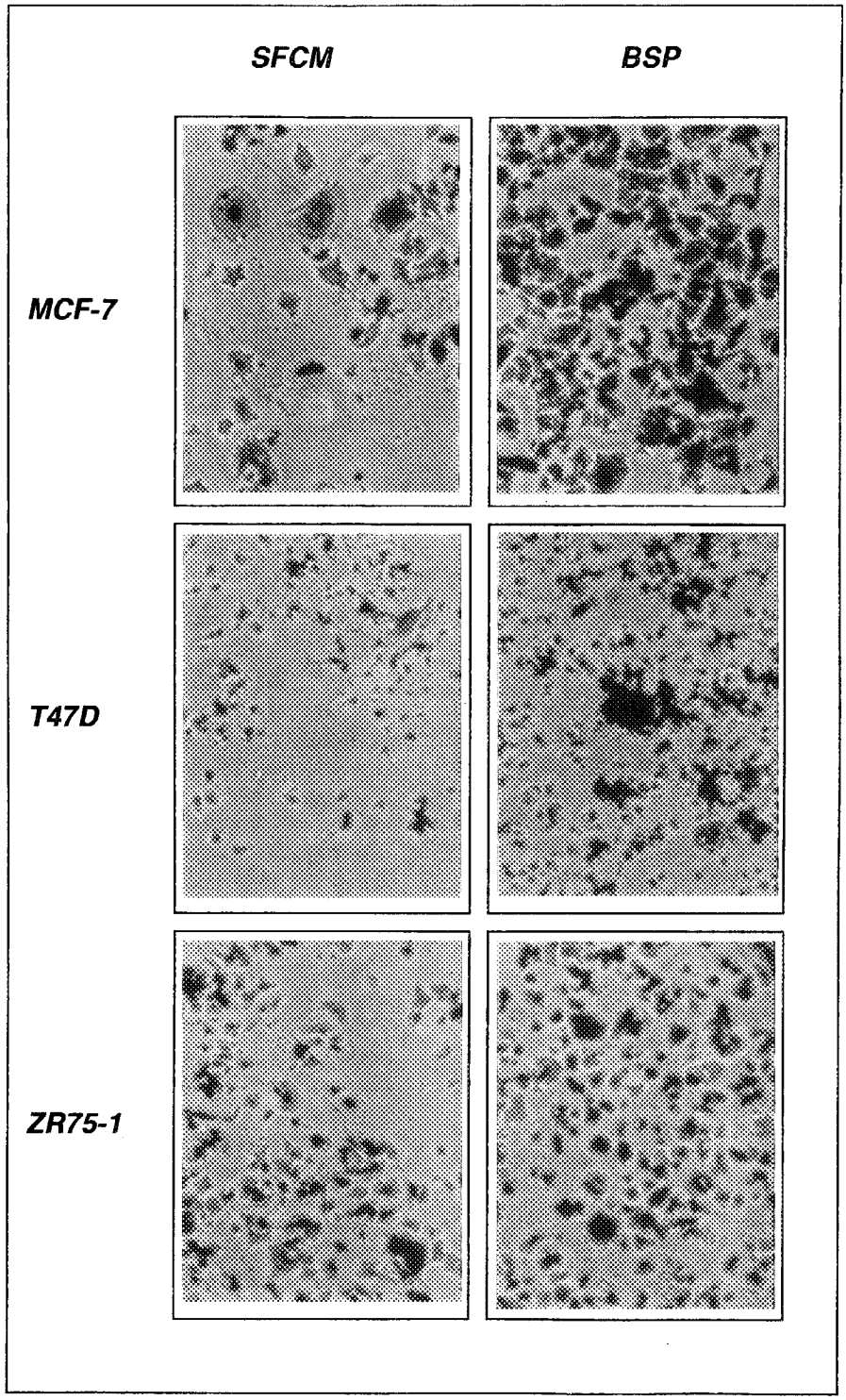

Figure 1.
Attachment of various breast cancer cells to serum-free conditioned medium (SFCM) of normal trabecular human bone cells and bone sialoprotein (BSP). Cells were fixed with 80% methanol after 3 hours of incubation and stained with Amidoblack. MCF-7 on SFCM (A) and BSP (B); T47D on SFCM (C) and BSP (D); and ZR75-1 on SFCM (E) and BSP (F). Original magnification, ×100.

investigated the qualitative and quantitative differenc-e(s) in integrin expression patterns among the tested breast cancer cell lines using immunoprecipitation with anti-integrin–specific antibodies. All tested breast cancer cell lines expressed various members of the integrin family (Fig. 2). However, differences existed among the cell lines. For instance, MDA-MB-231 cells expressed relatively high levels of β_1 and β_3 integrins, especially α_3, α_5, and $\alpha_v\beta_3$, when compared to the other tested cell lines (Fig. 2).

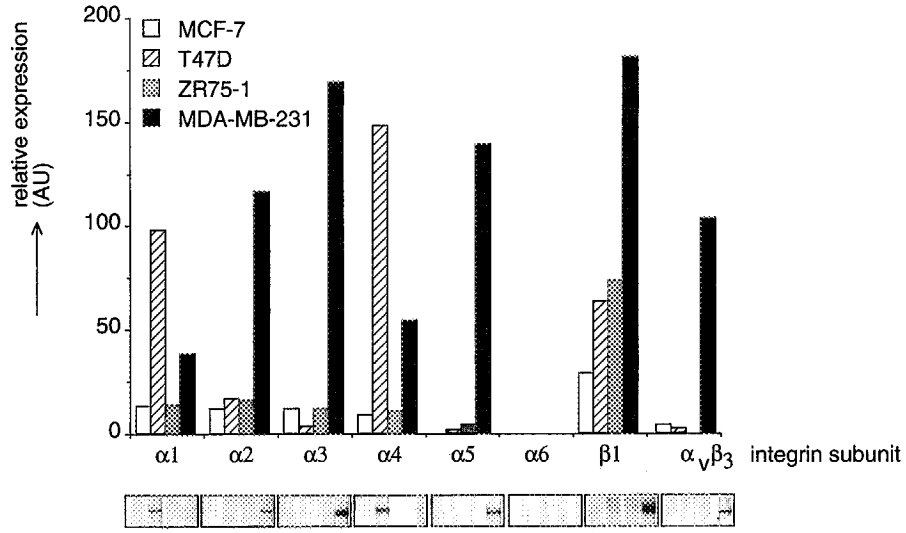

Figure 2.

Examples of qualitative and quantitative differences in α and β integrin (subunit) expression in tested breast cancer cell lines (MCF-7, T47D, ZR75-1, and MDA-MB-231) using immunoprecipitation as described in "Material and Methods." Relative expression among the cell lines was determined for each individual integrin subunit using a flatbed-scanner and image analysis software (see "Materials and Methods"). *AU* = Arbitrary units. Molecular weights of the integrins (in kilodaltons): $\alpha 1$ = 210, $\alpha 2$ = 165, $\alpha 3$ = 130, $\alpha 4$ = 150, $\alpha 5$ = 135, $\alpha 6$ = not detected, $\beta 1$ = 130, αv = 125.

Subsequently, we determined the contribution of various members of the integrin family in adhesion of MDA-MB-231 breast cancer cells to extracellular bone matrices. Function-blocking antibodies raised against β_1 and β_3 integrin heterodimers significantly inhibited adhesion of MDA-MB-231 cells to SFCM of human bone cells (Table 2). In particular, a panel of function-blocking anti-α_2, -α_3, -α_5, -$\alpha_v(\beta_3)$ integrin antibodies significantly blocked tumor cell adhesion to SFCM (Table 2).

Adhesion of breast cancer cells to trabecular bone matrix and involvement of integrins were investigated further in a recently described in vitro attachment assay (van der Pluijm et al, 1996a, 1996b), which comprises cryostat sections of developing trabecular bone (for description, see Figure 6 in "Materials and Methods" section). Breast cancer cells settled and spread on longitudinal cryostat sections of mouse tail vertebrae. Adhesion and spreading were most prominent on vertebrae, which consist of calcified cartilage and trabecular bone (Fig. 3, *open bars*). Attachment to more mature stages of vertebrae (vertebrae 21 and down) can be significantly inhibited with synthetic RGD peptides, as was expected (data not shown; van der Pluijm et al, 1996a). In the presence of a function-blocking monoclonal antibody against the β_1 integrin subunit (Fig. 3, *closed bars*), differences in the adhesion of breast cancer cells to tail vertebrae were found. Adhesion of MDA-MB-231 cells to hypertrophic cartilage was not inhibited by the β_1 integrin antibody. In contrast, adhesion of these cells to more mature stages of developing trabecular bone became increasingly susceptible to the anti-β_1 antibody, causing significant inhibition of adhesion (> 80%) at later developmental stages of trabecular bone.

Histologic sections of vertebrae showed that the breast cancer cells adhered to vertebrae with high affinity and subsequently spread in less than an hour (Fig. 4A). In the presence of an anti-β_1 integrin antibody (P4C10), however, adhesion of MDA-MB-231 cells to more mature vertebrae was strongly and significantly inhibited (> 80%; Fig. 4B). In addition, the decreased number of adherent cells were round-shaped, which is indicative of their inability to spread on the natural substrate (Fig. 4B). Interestingly, the anti-β_1 integrin antibody inhibited adhesion of tumor cells to surrounding tissue (skin and muscle), but the degree of inhibition was much less than that on vertebrae (± 50%; Fig. 4, C and D). Adhesion of breast cancer cells was significantly inhibited at more mature stages of developing trabecular bone with function-blocking monoclonal antibodies raised against α_2, α_3, α_5, $\alpha_v\beta_3$ integrins (Fig. 5), whereas isotypic, nonim-

Table 2. Inhibition of Tumor Cell[a] Adhesion (MDA-MB-231) to Extracellular Bone Matrix (SFCM)[b]

Control	α1	α2	α3	α4	α5	α6	αV	β1	β3
100 ± 5	110 ± 10	35 ± 7[c]	55 ± 6[c]	86 ± 6	55 ± 5[c]	80 ± 8	45 ± 2[c]	14 ± 3[c]	52 ± 5[c]

[a] Tumor cells were examined in the presence of function-blocking antibodies against various α and β integrin subunits.
[b] Data are expressed as % of control ± SEM (attached cells/area).
[c] $p \leq 0.05$.
Function blocking antibodies: α3 (P1B5), α4 (P4G9), α5 (BIIG2), α6 (135-13C), αv (13C2), β1 (P4C10), β3 (AB1932), and nonimmune IgG (control).

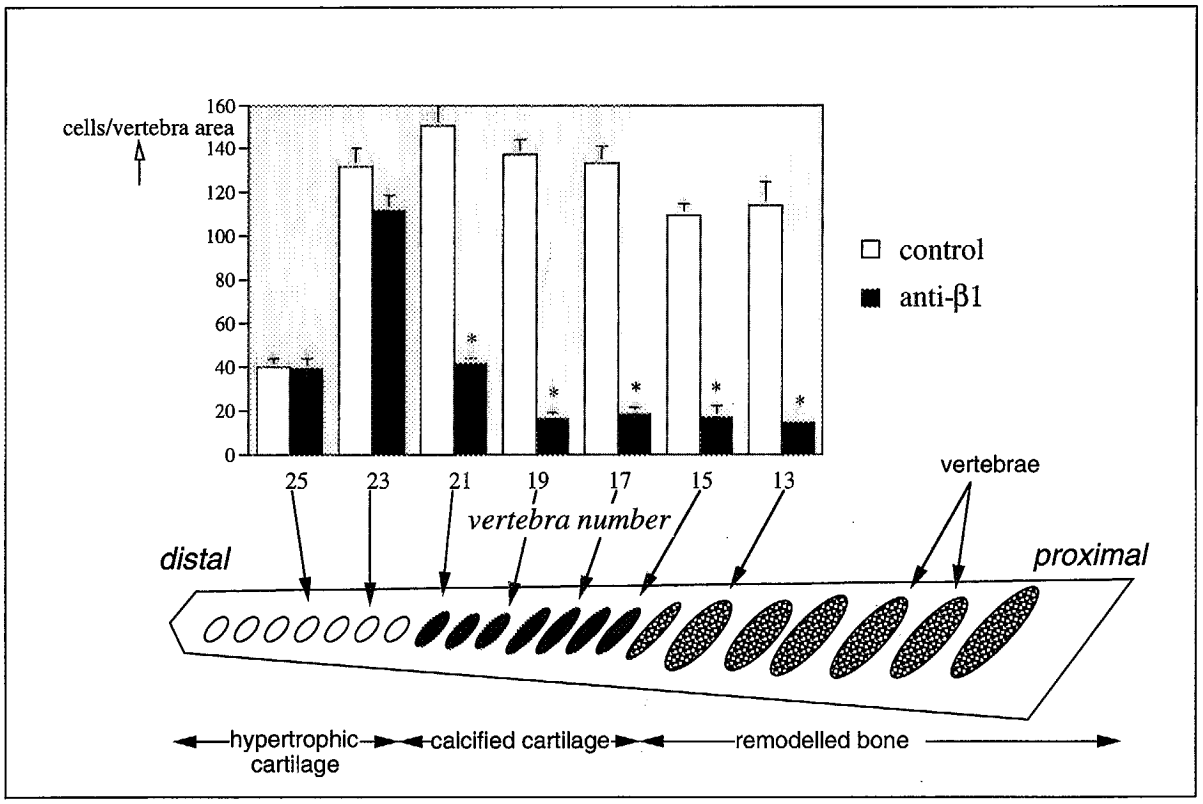

Figure 3.

Adhesion of MDA-MB-231 cells—in the presence (*closed bars*) or absence (*open bars*) of a 1:500 dilution of the function-blocking antibody against the $\beta 1$ integrin subunit (P4C10)—to cryostat sections of neonatal mouse tail vertebrae that each represent different developmental stages. The vertebrae of a 2-day old neonatal mouse tail can be divided into three developmental stages: caudal vertebrae 28 to 22 consist of noncalcified cartilage primordia, 21 to 15 of calcified hypertrophic cartilage, and 14 and down of mature remodeled trabecular bone, including bone marrow. A representative experiment is shown. The data are expressed as means ± SEM (cells/2.54 mm^2). The experiments were performed in 5-fold and repeated three times. Significance was calculated using a factorial one-way ANOVA followed by a Fisher's PLSD test.

mune IgG as well as α_4 and α_6 antibodies had no effect (data not shown).

Discussion

In this study, attachment characteristics of breast cancer cells to bone matrix constituents and putative involvement of integrins were determined. Our results show that breast cancer cells settle easily on extracellular bone matrix. In particular, MDA-MB-231 cells, which have been shown to metastasize to bone and to cause osteolytic lesions in nude mice (Sasaki et al, 1995; Yoneda et al, 1994; G van der Pluijm, unpublished observations), bind rapidly and in high numbers to extracellular bone matrix. In addition, MDA-MB-231 cells bind in larger numbers to a mixture of extracellular bone matrix proteins (SFCM of human trabecular bone cells) than to the individual proteins that contain the RGD sequence (OPN, BSP, FN, VN, COL-I, and TSP).

Quantitative and qualitative differences were found in integrin expression profiles among the tested breast cancer cell lines, which may explain the variations in adhesion characteristics. Tumorigenic MDA-MB-231 cells express high levels of β_1 and β_3 integrins, particularly COL/LAM ($\alpha_2\beta_1$,$\alpha_3\beta_1$), FN ($\alpha_5\beta_1$), and VN ($\alpha_v\beta_3$) receptors. Furthermore, α_2,α_3,α_5, $\alpha_v(\beta_3)$, β_1, and β_3 integrin (subunits) appear to be involved in adhesion of MDA-MB-231 cells to extracellular bone matrix. Results obtained in attachment assays with synthetic RGD peptides and integrin antibodies further support this notion.

In the mouse tail assay MDA-MB-231 cells adhere preferentially to more mature stages of trabecular bone. Again, adhesion of MDA-MB-231 cells to calcified cartilage and extracellular matrix of trabecular bone was significantly inhibited by function-blocking antibodies directed against α_2,α_3,α_5, $\alpha_v(\beta_3)$, β_1, and β_3 integrin (subunits). It appears, therefore, that osteogenic cells (eg, chondrocytes or osteoblasts) and/or bone marrow stromal cells, at more differentiated stages (caudal vertebrae 21 and up), start depositing extracellular matrix containing molecules with attachment properties. These newly synthesized matrix molecule(s) may, in turn, serve as ligands for adhesion receptors of the β_1 and/or β_3 integrin family on breast cancer cells.

It is important to note that all tested breast cancer cell lines bound strongly to OPN and BSP. These data are in accordance with a recently described, detailed, structure-function study of various synthetic BSP-

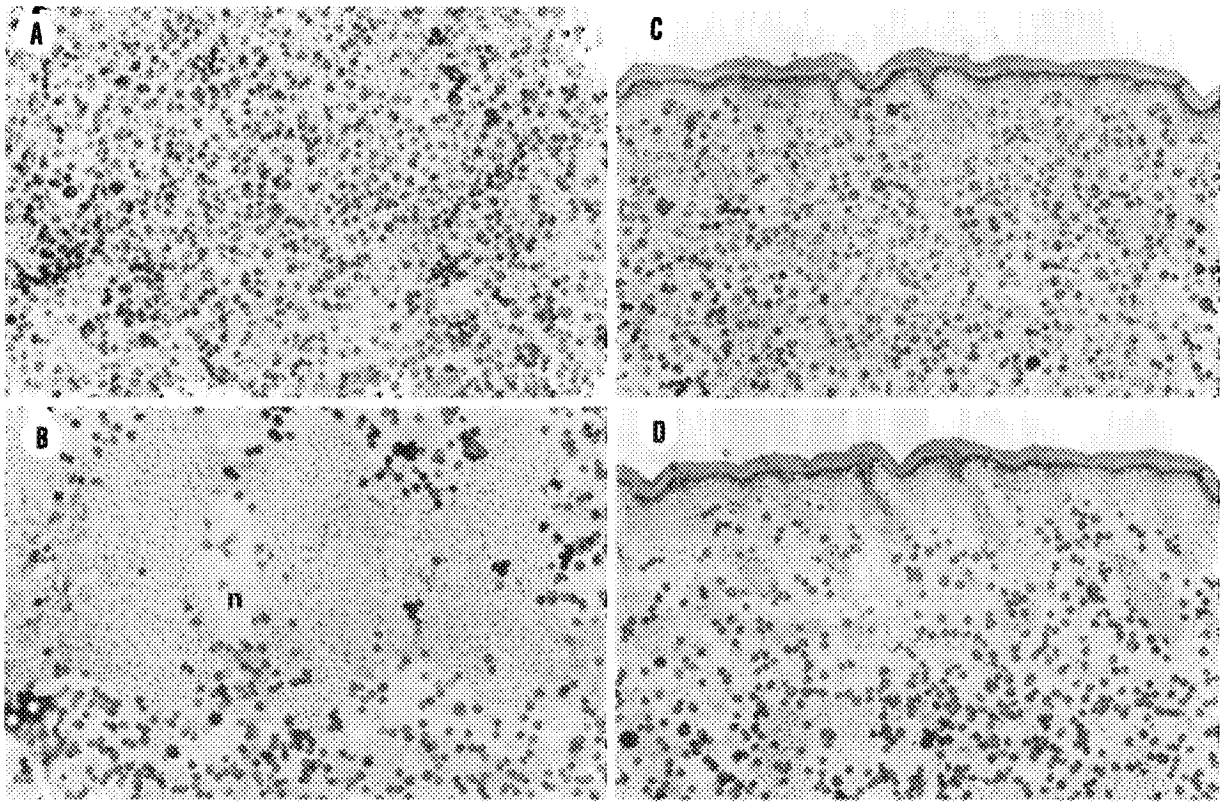

Figure 4.

Micrographs of the adhesion of breast cancer cells (MDA-MB-231) to neonatal mouse tail vertebrae (vertebrae 16 and 17; A and B) and surrounding tissue (C and D) in the absence or presence of function-blocking monoclonal antibodies against β_1 (P4C10). Cryostat sections were stained with hematoxylin (see "Materials and Methods"). Original magnification, ×100. Please note that no aspecific adhesion was found in the direct vicinity of the cryostat section. *N* = Nucleus pulposus of the intervertebral disc.

derived peptides (van der Pluijm et al, 1996a), where we showed that breast cancer cells adhere to synthetic BSP peptides that encompass the RGD motif and its flanking regions. Furthermore, exogenous addition of synthetic BSP peptides strongly and dose-dependently inhibited the adhesion of breast cancer cells to cortical and trabecular bone matrix (van der Pluijm et al, 1996a).

Recent evidence suggested that BSP is also produced by primary breast carcinomas (Bellahcène et al, 1994, 1996a, 1996b) and that the level of BSP expression in primary human breast cancer is associated with bone metastases development (Bellahcène et al, 1996a, 1996b). The frequency of bone metastases in a group of patients with BSP-positive tumors was significantly higher than in a group of patients with BSP-negative cancers. The authors hypothesized that BSP expression may be associated with poor prognosis and formation of bone metastases (Bellahcène et al, 1996a, 1996b). Furthermore, it has been suggested that changes in integrin receptor profiles in primary breast cancer may have an impact on their invasive and metastatic properties (Koukoulis et al, 1991; Natali et al, 1992; Oda et al, 1992; Pignatelli et al, 1992; Sager et al, 1993; Zutter et al, 1990). In addition, it has been suggested that bone metastases from breast cancer strongly express $\alpha_v\beta_3$ integrins (Kitazawa and Maeda, 1994; Liapis et al, 1996). The importance of these findings remains unclear, although it may be that expression of extracellular matrix molecules, such as BSP (Bellahcène et al, 1994, 1996a), by infiltrating breast cancer cells, and alteration in adhesion receptor expression profiles (Albelda, 1993; Gui et al, 1995; Juliano, 1993; Kitazawa and Maeda, 1995; Koukoulis et al, 1991; Liapis et al, 1996; Maemura and Dickson, 1994; Natali et al, 1992; Varner and Cheresh, 1996; Weaver et al, 1997; Zetter, 1993; Zutter et al, 1990) are prerequisites for the invasive behavior of these cells and for their ability to establish micrometastases in distant organs, including bone and bone marrow.

In conclusion, our findings suggest that COL/LAM ($\alpha_2\beta_1$,$\alpha_3\beta_1$), FN ($\alpha_5\beta_1$), and VN ($\alpha_v\beta_3$) receptors are mediators of the adhesion of breast cancer cells to extracellular bone matrix. It is tempting to speculate that β_1 and β_3 integrins may also be involved in the preferred homing of mammary carcinoma cells to the bone or bone marrow compartment. However, direct confirmation of such a role should await appropriate in vivo studies.

Materials and Methods

Breast Cancer Cell Lines

All four breast cancer cell lines (MCF-7, T47-D, ZR-75-1, MDA-MB-231) were purchased from the Amer-

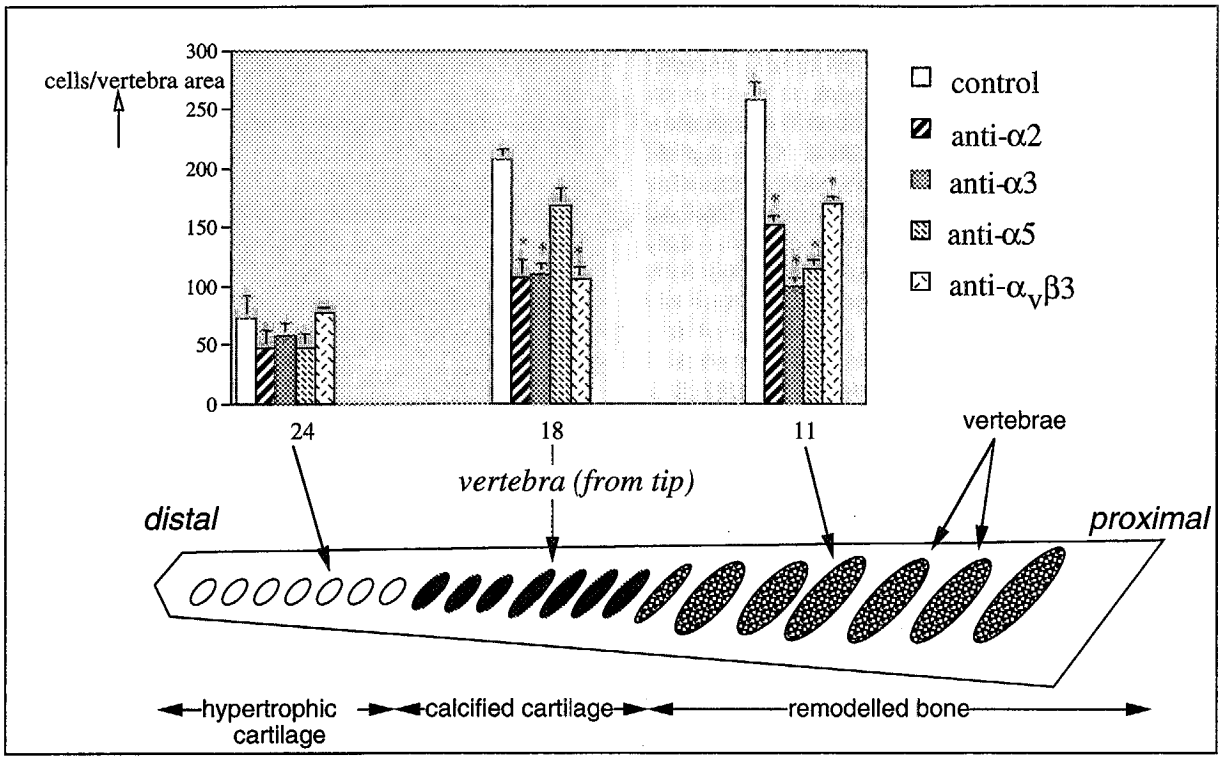

Figure 5.

Effects of function-blocking monoclonal antibodies against α_2, α_3, α_5, and α_v (β_3) on adhesion of MDA-MB-231 cells to mouse tail vertebrae 24 (noncalcified hypertrophic cartilage), 18 (calcified cartilage), and 11 (mature trabecular bone), each representing a different stage of trabecular bone development within one cryostat section. Antibodies used: nonimmune mouse IgG as control, 5E8(α_2), P1B5(α_3), P1D6(α_5), and 13C2(α_v). A representative experiment is shown. The data are expressed as means ± SEM (cells/2.54 mm^2). The experiments were performed in 5-fold and repeated three times. Significance was calculated using a factorial one-way ANOVA followed by a Fisher's PLSD test.

ican Type Culture Collection (Rockville, Maryland) (Cailleau et al, 1974). It has been shown that MDA-MB-231 cells, when injected into the left heart ventricle of nude mice, form osteolytic metastases (Sasaki et al, 1995; Yoneda et al, 1994; G van der Pluijm, unpublished observations).

Cells were cultured in $RPMI_{1640}$ + 10% FBS + P/S (Life Technologies–GIBCO-BRL, Gaithersburg, Maryland) in a humidified incubator at 37°C at 5% CO_2 until confluence. For attachment assays, tumor cells were cultured until approximately 90% confluence and dissociated into single-cell suspensions from the TC flasks using a 0.05% EDTA solution in PBS for 3 minutes (see "*Cell Attachment Assays*").

Matrix Glycoproteins and Reagents

Human VN and FN were purchased from Collaborative Research, Inc. (Bedford, Massachusetts). Bovine COL-I was purchased from Collagen Corporation (Palo Alto, California) and bovine serum albumin (BSA) from Miles Scientific, Inc. (Naperville, Illinois). Human TSP was purchased from Calbiochem (La Jolla, California). Rat BSP, isolated from UMR-106-BSP rat osteosarcoma cells (Mintz et al, 1993), was a gift from Dr. K. Mintz (National Institutes of Health, National Institute of Dental Research, Craniofacial and Skeletal Disease Branch [NIH-NIDR/CSDB], Bethesda, Maryland). Human OPN, which was isolated from human trabecular bone as previously described (Fisher, 1992; Fisher et al, 1983), was a gift from Dr. L. Fisher (NIH-NIDR/CSDB). Mouse LAM, isolated from mouse Engelbreth-Holm-Swarm tumors (Kleinman et al, 1986), was a gift from Dr. H. Kleinman (NIH-NIDR, Laboratory of Developmental Biology [LDB], Bethesda, Maryland). Serum-free conditioned medium (SFCM) was obtained from confluent, normal, primary human trabecular bone cells incubated for 24 hours in serum-free medium supplemented with 0.5% ITS^+ (insulin, transferrin, selenium, 2.5 μg/ml; bovine serum albumin (BSA), 0.5 mg/ml; and linoleic acid , 0.5 μg/ml; Collaborative Research, Inc.). The synthetic GRGDS peptide was purchased from Calbiochem, and cycloheximide was purchased from Sigma (St. Louis, Michigan).

Cell Attachment Assays

Proteins Coated onto Plastic. Cell attachment assays were performed in bacteriological 96-well plates, dotted with glycoproteins as described previously (Gehron Robey et al, 1989; Grzesik and Gehron Robey, 1994; van der Pluijm et al, 1996a, 1996b).

The putative role of integrins in tumor cells that recognize RGD motifs in their ligands was tested in the continuous presence of a high concentration of RGD

peptide (200 μg/ml GRGDS peptide). This concentration was found to be maximally effective for adhesion of susceptible tumor cells to RGD-containing extracellular matrix proteins (data not shown).

Adhesion of Breast Cancer Cells to Cryostat Sections of Developing Trabecular Bone. Two-day old neonatal tails were dissected, stretched in Tissue Tek (Miles Scientific), frozen in liquid N_2 for at least 10 minutes and stored at −80°C, as described previously (van der Pluijm et al, 1996a, 1996b). Five-μm sections were cut at −20°C using a cryostat (Microm HM 500 M; Microm GmbH, Heidelberg, Germany) and subsequently transferred to 3-amino- propyltriethoxysilane (Sigma)-coated glass coverslips and stored at −80°C until use.

Longitudinal sections (5 μM) cut through a 2-day-old neonatal mouse tail revealed three separate areas, each representing a specific maturational stage in development of trabecular bone (Fig. 6, A to C), starting from the distal end (early developmental stages) to the proximal site of the tail (remodeled trabecular bone). At the distal end (tail tip), there are noncalcified cartilage primordia (vertebrae 28 to 22), consisting mainly of hypertrophic cartilage, that will eventually develop into mature caudal vertebrae (Fig. 6A). In the midregion of the cryostat tail section, vertebrae 21 to 15 consist of calcified cartilage because remodeling has not yet occurred (Fig. 6B). At the proximal end of the tail, vertebrae (14 and down) consist of remodeled trabecular bone that may contain bone marrow (Fig. 6C).

Breast cancer cells were precultured in $RPMI_{1640}$-Dutch modification + 10% FBS + P/S in a humidified incubator at 37°C at 5% CO_2 until 90% confluence. For attachment assays, tumor cells were dissociated from the flasks using 0.05% EDTA solution in PBS (pH 7.2) for 3 minutes. Cells were washed and resuspended in serum-free medium, $RPMI_{1640}$ + 0.5% ITS^+. Tumor cells were resuspended, counted, and seeded at a density of 80,000 cells in 80 μl of medium onto longitudinal mouse tail sections in the presence or absence of function-blocking anti-integrin antibodies or RGD-peptides (see "*Antibodies Used in Attachment Assays . . .*") and incubated for 1 hour at 37°C in a humidified incubator (5% CO_2). After incubation, the sections were washed three times with PBS (to remove nonadherent cells), and attached cells were fixed in 4% paraformaldehyde for 30 minutes. Subsequently, the sections were washed with PBS and stained with hematoxylin. Cell numbers were determined by counting nonoverlapping microscopic fields for each central section of vertebra at an original 200 × magnification (area = 2.54 mm^2), as described previously (van der Pluijm et al, 1996a, 1996b).

Statistical Analysis

The experiments were performed in 5- or 6-fold and repeated three times. Significance was calculated using a factorial one-way ANOVA followed by a Fisher's PLSD test.

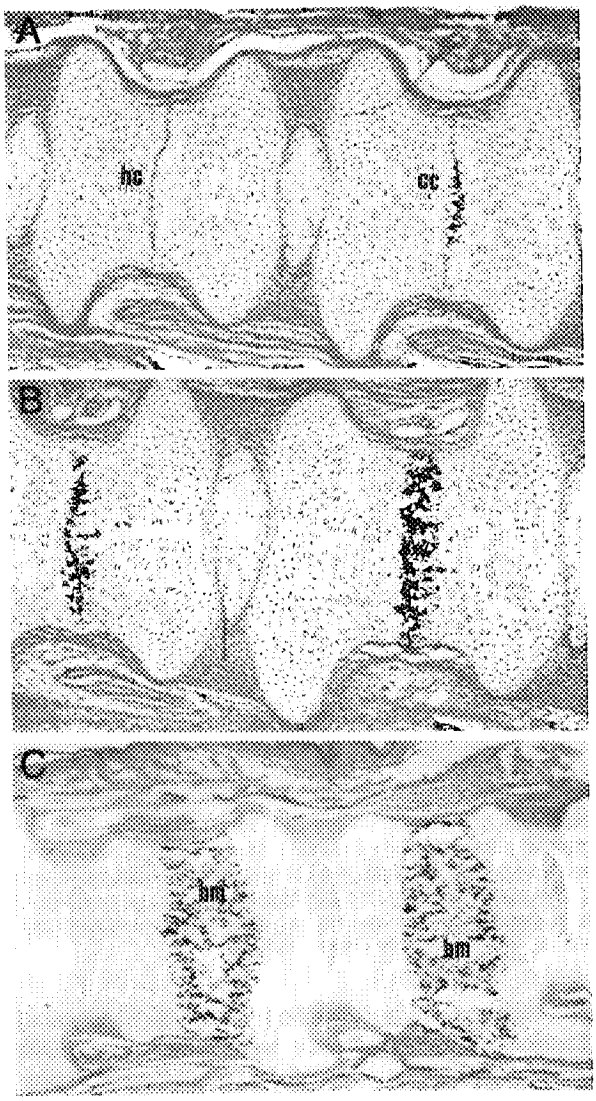

Figure 6.
Micrographs of three maturational stages of developing trabecular bone that can be identified within a longitudinal section of 2-day-old neonatal mouse tail. Three maturational stages of caudal vertebrae can be discriminated (from distal to proximal); hypertrophic cartilage (A), calcified cartilage (A and B), and remodeled trabecular bone with bone marrow (C). The tail sections were stained with von Kossa (1901). Original magnifications: ×100 (A and B), ×40 (C). *HC* = Hypertrophic cartilage of vertebra 22; *CC* = Calcified cartilage of vertebrae 21; *BM* = Bone marrow located within vertebrae 8 and 9.

Antibodies Used in Attachment Assays and Immunoprecipitation Experiments

The following function-blocking mouse monoclonal antibodies were used for attachment assays: anti-α_2, P1E6 (Life Technologies–GIBCO-BRL; Carter et al, 1990; Wayner et al, 1988)); anti-α_3, P1B5 (Life Technologies–GIBCO-BRL; Wayner et al, 1988); anti-α_4, P4G9 (Life Technologies–GIBCO-BRL; Wayner et al, 1989) and B-5G10 (kindly provided by Dr. M. Hemler, Dana-Farber Cancer Institute, Boston, Massachusetts); anti-α_5, P1D6 (Life Technologies–GIBCO-BRL; Wayner et al, 1988) and BIIG2 (kindly provided by Dr. C. Damsky, University of California, San Francisco, California; Brown et al, 1989; Werb et al, 1989); anti-α_v, 13C2 (kindly provided by Dr. M. Horton, Bone

and Mineral Centre, The Middlesex Hospital, London, United Kingdom; Horton et al, 1985); $\alpha_v\beta_3$, 23C6 (also provided by Dr. M. Horton; Davies et al, 1989); β_1, AIIB2 (also provided by Dr. C. Damsky; Brown et al, 1989) and P4C10 (Life Technologies–GIBCO-BRL; Carter et al, 1990). A function-blocking rabbit polyclonal antibody against β_3, AB1932, was also used (Chemicon International, Inc., Temecula California).

In addition to the antibodies listed above, the following (non–function-blocking) mono- and polyclonal antibodies were used in immunoprecipitation experiments: α_1, AB1934 (Chemicon) and TS2/7(also provided by Dr. M. Hemler; Hemler, 1990; Hemler et al, 1984); anti-α_2, 5E8 (kindly provided by Drs. Bankert and Chen, Roswell Park Cancer Institute, Buffalo, New York; Zylstra et al, 1986) and AB1936 (Chemicon); anti-α_3, AB1920 (Chemicon) and J143 (kindly provided by Dr. T. Albino, Memorial Sloan Kettering Institute, New York, New York); anti-α_4, AB1924 (Chemicon); anti-α_5:, Ab16 (kindly provided by Drs. S. and K. Yamada (NIH-NIDR/LDB; Akiyama et al, 1989) and AB1928 (Chemicon); anti-α_6, 135-13C (kindly provided by Dr. S. J. Kennel, Oak Ridge National Laboratory, Memphis, Tennessee; Kennel et al, 1981); anti-α_v, AB1930 (Chemicon); anti-β_1, AB1938P (Chemicon), TS2/16 (also provided by Dr. M. Hemler; Hemler et al, 1984), AIIB2 (also provided by Dr. C. Damsky; Brown et al, 1989), and mAb 13 (also provided by Drs. S. and K. Yamada, NIH-NIDR/LDB; Akiyama et al, 1989). Isotypic, nonimmune Ig or normal sera were used as negative controls during immunoprecipitation and adhesion experiments.

Integrin Expression by Breast Cancer Cells

Breast cancer cell lines were plated at different densities and cultured for 48 hours in 6-well dishes with 1.5 ml $RPMI_{1640}$ medium (without leucine/proline) (GIBCO-BRL, Grand Island, New York) containing 10% dialyzed FCS (GIBCO-BRL) and 5 μCi ^{14}C-leucine/proline-translabel (Amersham, Arlington Heights, Illinois). Different initial densities for the cell lines were used to harvest cells for immunoprecipitation procedures in identical (exponential) growth phase (~80.000 cells/cm^2). In a parallel experiment, using 24-well plates, cell density was determined using a Coulter counter (Coulter Corporation, Miami, Florida). These data were used to normalize the amount of radiolabeled cell extracts for quantitative immunoprecipitations. Simultaneously, the cell layer was harvested with a rubber policeman in immunoprecipitation buffer (0.1 M Tris HCl, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, 1% Aprotinin, 1% deoxycholate [pH 7.2]), as described previously (Gehron Robey et al, 1989, Grzesik and Gehron Robey, 1994). Normal serum and specific antibody resin was prepared with (1:10 diluted) protein G sepharose (PGS) in PTA buffer (10 × PBS, 0.5% triton X-100, 0.05% BSA-fraction V). The immunoprecipitation procedure was carried out in three subsequent steps: PGS sweep, normal serum sweep, and specific antibody sweep. After the PGS and normal serum sweeps were performed, the supernatant was used for the specific antibody sweep, and the pellets were washed three times in PTA buffer and used as negative/aspecific controls. After the antibody sweep, the supernatant was discarded and the pellet was washed three times. Pellets (both normal serum and specific antibody) were resuspended in 2× gelsample buffer with DTT. Samples were boiled for 5 to 10 minutes and centrifuged, and 30 μl of the supernatant was run, after normalization for cell numbers, on 4% to 20% SDS-PAGE gels. The gels were fixed, enhanced in PPO/DMSO, dried, and exposed to Kodak XoMAT x-ray film at −70°C. Relative expression among the cell lines was determined for each individual integrin subunit using a flatbed-scanner (Umax Powerlook II; Umax Data Systems, Inc., Taiwan, China) and image analysis software (NIH-Image 1.60/ppc). Data are expressed as arbitrary units.

Tumor Cell Adhesion and Concentration of Coated Extracellular Matrix Components

A broad concentration range of various extracellular matrix components (0.0001 to 2 μM) was tested for ability to induce adhesion of all tested breast carcinoma cell lines after 3 hours of incubation. Maximal cell attachment for all tested cell lines, expressed as % of attached cells (100% = 10,000 cells/cm^2), was generally reached at extracellular matrix protein concentrations higher than 0.002 μM for COL-I, OPN, BSP, FN, VN, TSP, and LAM (data not shown).

Plastic coated with SFCM of human bone cells (1:1 to 1:1000 dilution in PBS + 1 mM Ca^{2+}) strongly promoted the adhesion of all breast cancer cell lines tested. Breast cancer cell adhesion to SFCM of human bone cells reached a maximum at 1:10 dilution. Based on these observations, 0.2 μM of extracellular matrix proteins and 1:1 dilution of SFCM of human bone cells are used for all adhesion experiments described in the present paper. Maximal adhesion of different cell lines to SFCM of normal human trabecular bone cells was generally reached within 3 hours of incubation (data not shown).

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